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In Vitro and in Vivo Antiestrogenic Effects of Polycyclic Musks in Zebrafish

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The polycyclic musks 6-acetyl-1,1,2,4,4,7-hexamethyltetraline (AHTN) and 1,2,4,6,7,8-hexahydro-4,6,6,7,8,8-hexamethylcyclopenta-γ-2-benzopyran (HHCB) are used as fragrance ingredients in perfumes, soaps, and household cleaning products. They are known to be ubiquitously present in the aquatic environment, and because of their lipophilic nature, they tend to bioaccumulate in aquatic biota. In surface waters, concentrations between 1 ng/L and 5 μg/L have been found, depending mainly on the proportion of sewage effluents in the water. In fish, under normal environmental conditions, concentrations in the microgram per kilogram fresh weight (fw) range are found. In a previous study we showed that AHTN and HHCB exert mainly antiestrogenic effects on the human estrogen receptor α (ERα) and ERβ in an in vitro reporter gene assay. In the current study, we assessed the in vitro antiestrogenic effects of both musks on zebrafish ERs. Antagonism was observed on zfERβ, and more pronounced on the newly cloned zfERγ. Using a transgenic zebrafish assay, we studied antiestrogenicity of the musks in vivo. Dose-dependent antagonistic effects were observed at concentrations of 0.1 and 1 μM AHTN and HHCB. GC−MS analysis showed that the musks bioaccumulated in the fish, with internal concentrations (15−150 mg/kg fw) which were roughly 600 times higher than the nominal test doses. To our knowledge, this is the first time that environmental contaminants are shown to be antiestrogenic in an in vivo fish assay that focuses solely on ER-mediated effects. This makes the transgenic zebrafish assay a promising tool for the rapid detection of both estrogenic and antiestrogenic effects of chemicals in fish.

Introduction

Two chemicals that are ubiquitously present in the aquatic environment are the polycyclic musks 6-acetyl-1,1,2,4,4,7-hexamethyltetraline (AHTN) and 1,2,4,6,7,8-hexahydro-4,6,6,7,8,8-hexamethylcyclopenta-γ-2-benzopyran (HHCB). Polycyclic musks are used as fragrance compounds in laundry detergents, soaps, and cosmetics, with a worldwide production volume of about 6000 tons per year (*1*). They may reach the aquatic environment via wastewater treatment plants, and consequently, because of their lipophilic character, they tend to bioaccumulate in fish and other aquatic organisms. Log $K_{o/w}$ values of 5.7 and 5.9 have been found for AHTN and HHCB, respectively (*1*). Concentrations of HHCB vary from 1 ng/L in clean surface waters up to 5 μg/L in surface water with a high proportion of effluents of sewage treatment plants (*2, 3*). In sewage water effluents concentrations of AHTN and HHCB were found up to 4 and 13 μg/L, respectively (*3*). Environmental concentrations of AHTN are usually lower, reflecting its lower use volume (*4*). In eel (*Anguilla anguilla*) samples from lakes with a high input of sewage water, AHTN and HHCB concentrations were found up to 3 and 6.5 mg/kg of lipid (0.7 and 1.5 mg/kg fresh weight (fw)), respectively, with maximal concentrations which are 3 times higher (*3*). In other fish species lower concentrations were found, due to their lower fat content. Also in surface waters with a low or moderate input of sewage water, lower concentrations in the microgram per kilogram fw range were found.

Surprisingly, despite the fact that polycyclic musks are ubiquitously present in the aquatic environment and tend to bioaccumulate in aquatic biota, little is known of their ability to disrupt endocrine systems, such as estrogen homeostasis. Using cell lines stably transfected with an estrogen-responsive reporter construct and the human estrogen receptor α (ERα) or ERβ, we recently found that both polycyclic musks show antiestrogenic and weak estrogenic effects, depending on the cell line and ER subtype used (*5*). In the present study, we were interested to assess the (anti)estrogenic effects of these compounds in fish. Examples can be found of wild fish populations showing disturbances of normal endocrine functions, and occurrences of intersex and testis abnormalities (*6*−*8*). These effects have been associated with the exposure to natural, synthetic, and xenoestrogens in the aquatic habitats. Here, we assess the (anti)estrogenic effects of AHTN and HHCB on the zebrafish ERα, ERβ, and the recently cloned ERγ (*9*), which is also indicated as ERβ2 (*10, 11*). The experiments show no agonism of the musks on all three receptors, but antagonism on zfERβ and zfERγ. Furthermore, we investigated the antiestrogenic effects in vivo using a transgenic zebrafish assay (*12*), and we correlated these effects to the measured internal dose. Both AHTN and HHCB showed dose-dependent antagonistic effects at test concentrations of 0.1 and 1 μM, and internal doses which are roughly 600 times higher. As far as we know, in vivo antiestrogenic effects of environmental contaminants in fish have never been described before in the scientific literature.

Materials and Methods

Chemicals and Reagents. 17β-Estradiol was purchased from Sigma (St. Louis, MO). HHCB and AHTN were kind gifts from International Flavours and Fragrances (IFF), Hilversum, The Netherlands, and PFW-Aroma Chemicals, Barneveld, The Netherlands, respectively. 4-Hydroxytamoxifen was a kind gift from Dr. A. Wakeling (Zeneca Pharmaceuticals, Macclesfield, U.K.).

Cell Culture. Human embryonal kidney 293 (HEK293) cells were obtained from the American Type Culture Collection (ATCC; Rockville, MD). Cells were maintained in a 1:1 mixture of Dulbecco's modified Eagle's medium (DMEM)

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and Ham's F12 medium (DF; Life Technologies Inc., Gaithersburg, MD) supplemented with 7.5% fetal calf serum (FCS) (Integro, Linz, Austria). The cell line was cultured at 37 °C and 7.5% CO_2.

Plasmids. The human ERα expression plasmid pSG5-HEGO was kindly provided by Dr. P. Chambon (IGBMC, Strasbourg, France). The human ERβ expression plasmid pSG5-hERβ was kindly provided by Dr. J.-Å. Gustafsson (Karolinska Institute, Huddinge, Sweden). The estrogen-responsive reporter gene construct (3xERE-TATA-Luc), which contains three copies of a consensus estrogen response element (ERE) containing oligonucleotide and a TATA box in front of the luciferase cDNA, is described in more detail elsewhere (*13*). The cloning of the full-length zebrafish (zf) ERα, zfERβ, and zfERγ and the insertion into the *Eco*RI site of the multiple cloning site of the pSG5 expression vector (Stratagene) are described elsewhere (Zeinstra et al., manuscript in preparation).

Transient Transfections. HEK293 cells were plated in 800 μL of phenol red-free DF medium containing 30 nM selenite, 10 μg/mL transferrin, and 0.2% BSA, supplemented with 5% dextran charcoal stripped FCS, at 8×10^4 cells per well of a 24-well plate (Costar). Cells were transfected using the calcium phosphate precipitation method (*14*) 30 h after plating.

A total amount of 2.0 μg of DNA/well was transfected, consisting of 0.6 μg of luciferase reporter plasmid, 0.6 μg of PDM-LacZ internal control plasmid, 0.6 μg of pBluescript SK^-, and 0.2 μg of ER expression plasmid. After an overnight incubation cells were given fresh media with or without test compounds (dissolved in ethanol, maximum 0.1% solvent).

After 24 h, the cells were lysed in 200 μL of lysis solution (1% Triton X-100, 25 mM glycylglycine, 15 mM $MgSO_4$, 4 mM EGTA, and 1 mM DTT). A 50 μL portion of cell lysate was transferred to a black 96-well plate to which 50 μL of luciferine substrate (LucLite reporter gene assay kit, Packard Instruments, Meriden, CT) was added. Luciferase activity was measured in a Topcount liquid scintillation counter (Packard Instruments) for 0.1 min per well. To correct for variations in transfection efficiencies, β-galactosidase activity was measured (*15*).

Transgenic Zebrafish Assay. The development of the transgenic zebrafish assay is described elsewhere (*12*). Exposure studies were carried out with homozygous F4 juvenile fish of 4−5 weeks of age. Fish ($n = 5-6$) were exposed for 96 h in 200 mL of acclimated tap water (26−27 °C) in beaker glasses. The compounds to be tested (dissolved in DMSO) were added to the water in a 1:10000 dilution. Fish were fed once daily with live brine shrimp (*Artemia salinas*). Half of the test medium was renewed daily. At the end of the exposure, fish were sacrified in ice−water, transferred to Eppendorf vials, and immediately frozen at −80 °C. To assay luciferase activity, Eppendorf vials containing whole fish were transferred to ice, 500 μL of cold lysis solution (1% Triton X-100, 25 mM glycylglycine, 15 mM $MgSO_4$, 4 mM EGTA, and 1 mM DTT, pH 7) was added, and the fish were homogenized using a micropestle (Eppendorf). After centrifugation at 12000 rpm for 15 min at 4 °C, duplicate samples of 50 μL of supernatant were transferred to a black 96-well plate (Packard) to which 50 μL of luciferine substrate (LucLite reporter gene assay kit, Packard) was added. Luciferase activity was measured in a scintillation counter (Packard Topcount) for 0.1 min per well.

Internal Exposure. To assess the internal concentration of both musks in the zebrafish, six animals were exposed to 0.1 or 1 μM (i.e., 25.8 or 258 μg/L) musk for 1, 2, 3, or 4 days. Fish were exposed in 200 mL of acclimated tap water (26−27 °C) in beaker glasses. The compounds to be tested (dissolved in DMSO) were added to the water in a 1:10000 dilution. Fish were fed once daily with live brine shrimp (*A. salinas*). Each day, before half of the test medium was refreshed, water samples were taken. After exposure, fish were sacrificied, and cleaned up for GC−MS analysis. The same exposure experiment was done in the absence of zebrafish.

Extraction and Cleanup of Fish Samples and Water Samples. Fish sacrificed on ice were spiked with 1 μg of AHTN or HHCB as an internal standard, and ultrasonicated in a mixture of 10 mL of water and 4 mL of cyclohexane. Then 4 g of dry NaCl was added, and after centrifuging, the upper cyclohexane layer was transferred into a glass test tube. Again 4 mL of cyclohexane was added to the sample, and the procedure was repeated. After concentration under nitrogen to 1 mL, the organic layer was eluted on a silica column using a 6 mL mixture of cyclohexane and ethyl acetate (98:2 v/v). The eluate was concentrated under nitrogen and transferred into a vial for GC−MS analysis.

Water samples of 3 mL were extracted using 4 mL of cyclohexane. The organic layer was spiked with either 1 μg of AHTN or HHCB, concentrated under nitrogen, and transferred into a vial for GC−MS analysis.

GC−MS Analysis. AHTN and HHCB concentrations in all extracts were analyzed on a Carlo Erba 5300 GC (Milan, Italy) equipped with a split/splitless injector, a 30 m × 0.25 mm (0.25 μm film thickness) fused silica DB-5MS column (J&W Scientific, Folson, CA), and a QMD 1000 mass spectrometer (Carlo Erba Instruments, Milan, Italy). Analyses were carried out by splitless injection of 1 μL at 225 °C. The column temperature was maintained at 90 °C for 1 min, and raised by 30 °C/min to 150 °C followed by 4 °C/min to 210 °C. The mass spectrometer was operated using selected ion monitoring (SIM), for m/z 243. Unknown concentrations were quantified with the peak area ratio of the compound to be quantified and the internal standard, using a standard curve. The recovery of this method is 100%.

Data Analysis. Luciferase activity per well was measured as light units. In the in vitro experiments, each concentration was analyzed in triplicate. The luciferase activity per well was divided by the concomitant β-galactosidase activity. In the transgenic zebrafish assay five to six fish per concentration were measured. From these values, the fold induction was calculated by dividing the mean value in exposed and nonexposed wells or fish. ERE-luc activity as a percentage of estradiol induction is calculated by setting the fold induction of estradiol at 100%. Data are mean values ± SEM from at least three independent in vitro experiments or from 5−36 transgenic zebrafish. The EC_{50} values for the transient transfections were calculated from sigmoidal dose−response curves using the curve-fitter of GraphPad Prism 3.0. The R of the fit of the curves was > 0.99. EC_{50} values were calculated by determining the concentration at which 50% of the maximal luciferase activity was reached. An unpaired Student's t test was used to compare differences between mean values of two different treatments. Data for dose−response studies were analyzed for statistical significance by one-way ANOVA and a least significant difference (LSD) test. Differences of $P < 0.05$ were accepted as statistically significant.

Results

Human and Zebrafish ER Transactivation by Estradiol in a Transiently Transfected HEK293 Reporter Cell Line. To compare the human and zebrafish ERs for E2-induced transcriptional activation, we used the HEK293 cell line. This cell line lacks significant endogenous levels of ER, can be easily transfected, and has been shown to be highly responsive to estrogens in transient transfections (*16*). Cells were transiently transfected with one of the tested ER expression plasmids and a reporter construct, consisting of three EREs upstream from a TATA box in front of luciferase cDNA.

Dose−response curves of E2 are shown in Figure 1. Transcriptional activity at hERα was about 1 order of

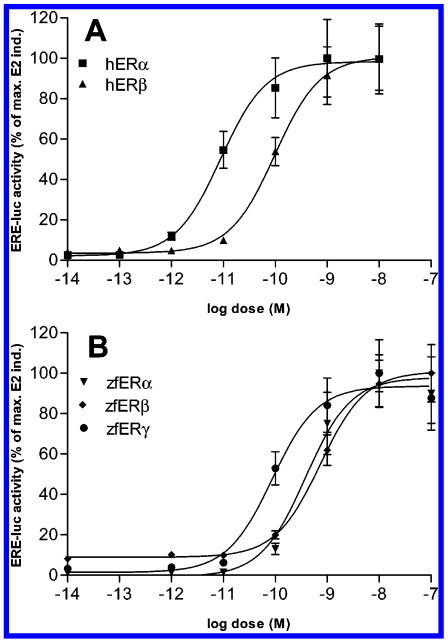

FIGURE 1. Activation of transcription of hERα and hERβ (A) and zfERα, zfERβ, and zfERγ (B) by 17β-estradiol in transiently transfected HEK293 cells. Results are expressed as a percentage of maximal E2 induction of each receptor subtype. Values represent means ± SEM from three independent experiments with each concentration measured in triplicate.

magnitude higher than that of hERβ (Figure 1A), with EC_{50} values of 0.009 and 0.10 nM, respectively. When the zebrafish ERs were compared, ERγ was the most responsive to E2, suggesting an important role in estrogen regulation (Figure 1B). Zebrafish ERα and ERβ were about equally sensitive, but less sensitive than zfERγ. We found EC_{50} values of 0.38, 0.73, and 0.09 nM for zfERα, zfERβ, and zfERγ, respectively. Comparison of human and zebrafish ERs revealed that E2-induced transcriptional activity of the human ERα was about 40 times higher than its zebrafish counterpart, while human ERβ was only about 7 times more responsive than zfERβ. Together with the small difference in sensitivity between zfERα and zfERβ, in contrast to the human ERs, these findings may suggest that zfERβ, compared to its ERα counterpart, is relatively more sensitive to estrogens than hERβ. These findings are consistent with another study (9). In addition, we used the dose−response curves to determine the submaximal E2 dose for each receptor subtype. At this concentration the response can be influenced most sensitively and can therefore be used in competition experiments, to assess antiestrogenicity.

Inhibition of E2-Induced Transactivation of Human and Zebrafish ER by AHTN and HHCB. To assess the antiestrogenic effects of AHTN and HHCB, we dosed the transiently transfected HEK293 cells with different concentrations of musk, and a submaximal concentration of estradiol. A submaximal dose of 0.01 nM E2 was used for hERα-mediated experiments, a dose of 0.1 nM was used for hERβ and zfERγ, and 1 nM E2 was used for zfERα and zfERβ. AHTN and HHCB alone showed a marginal transcriptional activation of the human ERα, at the highest test concentration of 10 μM. This was also previously shown in stably transfected HEK293 cells (5). Neither of the musks could stimulate transcriptional activity of the zebrafish ERs and the human ERβ (data not shown). As a positive control for antiestrogenicity in this experiment, the well-known antiestrogen 4-hydroxytamoxifen (OHT) was used at a concentration of 0.01 μM. OHT strongly inhibited the E2-stimulated transcriptional activation of all tested estrogen receptors (Figure 2). A clear dose-dependent and significant suppression of E2

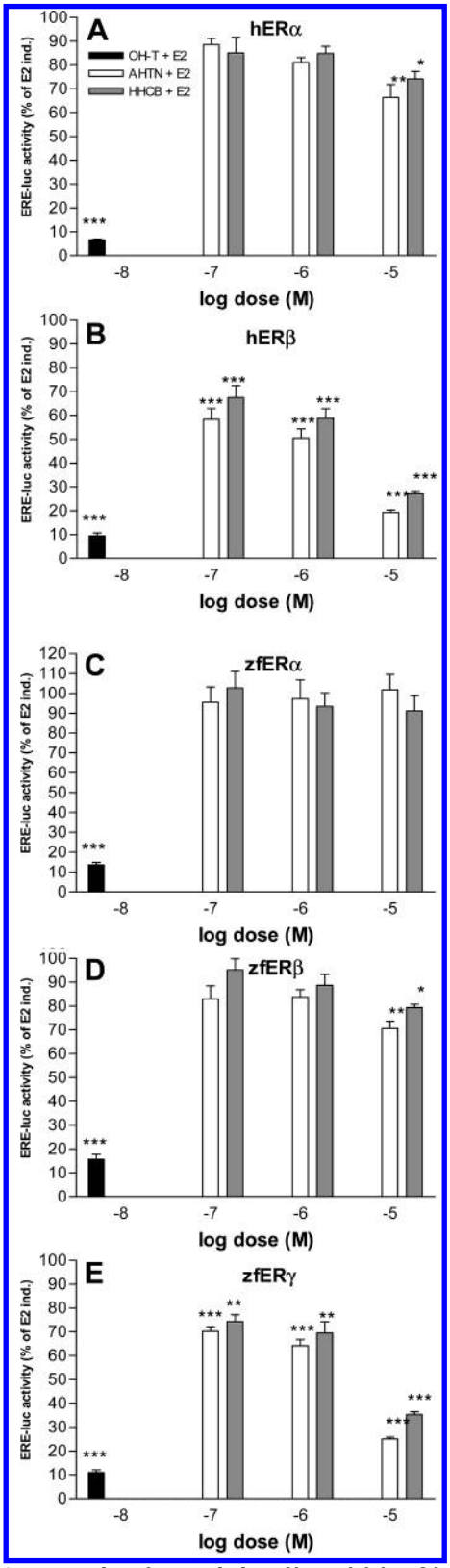

FIGURE 2. Repression of transcription of hERα (A), hERβ (B), zfERα (C), zfERβ (D), or zfERγ (E) by OH-tamoxifen (black bar; 0.01 μM), AHTN (white bars; 0.1−10 μM), and HHCB (gray bars; 0.1−10 μM) in transiently transfected HEK293 cells. Results are expressed as a percentage of submaximal E2 induction of each receptor subtype (hERα, 0.01 nM; hERβ, 0.1 nM; zfERα, 1 nM; zfERβ, 1 nM; zfERγ, 0.1 nM). Values represent means ± SEM from three independent experiments with each concentration measured in triplicate. Key: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ (by one-way ANOVA and LSD for differences between E2 treatment alone and E2 + compound).

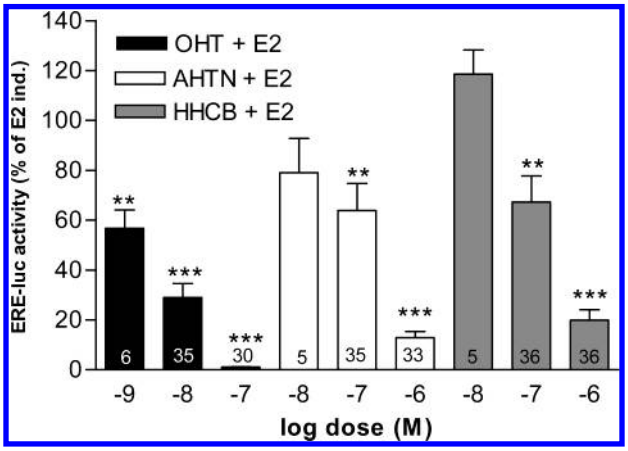

FIGURE 3. Luciferase activity in juvenile transgenic zebrafish exposed for 96 h to 10 nM estradiol together with OHT (black bars; 1−100 nM), AHTN (white bars; 0.01−1 μM), or HHCB (gray bars; 0.01−1 μM). Results are expressed as a percentage of E2 induction. Values represent means ± SEM from 5−36 fish. The number in the bar represents the number of fish tested. Key: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ (by one-way ANOVA and LSD for differences between E2 treatment alone and E2 + compound).

induction by AHTN and HHCB was shown toward human ERβ and zebrafish ERγ (Figure 2B,E). A weak antagonistic effect could be observed on human ERα and zfERβ only at the highest test concentration of 10 μM (Figure 2A,D), whereas no effect was seen at zfERα (Figure 2C). Coadministration of the musks with higher concentrations of E2 abolished the antagonistic effects of the musks, which suggests a competitive interaction at the level of the ER (data not shown).

Inhibition of E2-Induced Transactivation by AHTN and HHCB in Transgenic Zebrafish. Since both musks antagonized E2-induced transcription on the zebrafish ERβ and ERγ in vitro, we wished to examine whether these antiestrogenic effects could also be observed in vivo. Therefore, we used a transgenic zebrafish assay, a rapid and specific in vivo assay for the detection of (xeno)estrogens (*12*). In these zebrafish, the same reporter gene construct was stably introduced as the one that was used in the HEK293 transient transfections. High responsiveness has been shown to natural and synthetic (xeno)estrogens, such as estradiol, estrone, DES, ethinyl estradiol, and DDT (*12*). The detection limit of this assay is about 0.3 nM E2, and the EC_{50} is about 10 nM E2 (*9*). When AHTN or HHCB was tested, concentrations of 10 μM were toxic to the fish. Both AHTN and HHCB did not show any estrogenic effect in this in vivo assay (data not shown).

We assessed for antiestrogenicity at the EC_{50} of the dose−response curve of E2, namely, at 10 nM E2. At this concentration, the positive control OHT showed a clear inhibition of E2-stimulated transactivation (Figure 3). Both HHCB and AHTN showed dose-dependent antagonistic effects at concentrations of 100 and 1000 nM. The antiestrogenic effects were still present at 100 nM E2, the maximum of the dose−response curve, but less pronounced (data not shown). Compared to OHT, the musks were about 2 orders of magnitude less effective in reducing luciferase activity.

GC−MS Analysis of AHTN and HHCB during the Transgenic Zebrafish Assay. Fish were exposed to 0.1 or 1 μM (i.e., 25.8 or 258 μg/L) musk for 1, 2, 3, or 4 days. Each day, before half of the test medium was refreshed, water samples were taken. Results are shown in Table 1. Both in the absence and in the presence of zebrafish, immediately after dosing ($t = 0$), 10−35% of the musk has disappeared from the water, either by absorption at the glass or by diffusion into the air. At the end of the 96 h exposure without zebrafish, water concentrations were 25−40% lower than the nominal concentration. In the presence of fish, concentrations in the water at the end of the 96 h exposure were about 1.5 and 13 μg of AHTN/L and 2.9 and 15 μg of HHCB/L, for the low and high doses, respectively. These concentrations were reached within 24 h, and remained essentially unchanged thereafter.

TABLE 1. Concentrations of AHTN and HHCB Determined in Test Water during the Internal Exposure Experiment[a]

		water concn (μg/L)					
		absence of fish			presence of fish		
compd	nominal concn	$t = 0$ h	$t = 24$ h	$t = 96$ h	$t = 0$ h	$t = 24$ h	$t = 96$ h
AHTN	25.8	18	17	15	18	1.3	1.5
	258	229	232	196	227	14	13
HHCB	25.8	22	18	16	20	2.7	2.9
	258	176	173	158	169	17	15

[a] See Materials and Methods for details. Concentrations are expressed as micrograms per liter.

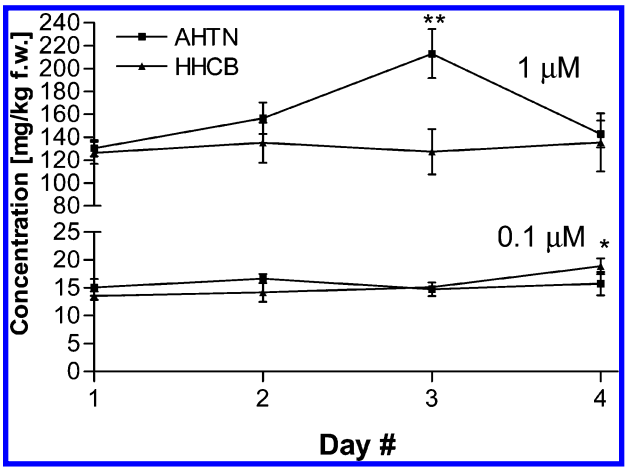

FIGURE 4. Internal concentrations of AHTN and HHCB at nominal test concentrations of 0.1 (bottom part of graph) and 1 μM (top part of graph) during the 4-day internal exposure experiment. Results are expressed as milligrams of musk per kilogram fresh weight. Values represent means ± SEM from 5−6 fish. Key: *, $P < 0.05$; **, $P < 0.01$ (by Student's *t* test for differences between day 1 and the following days).

We found that the internal concentration also stayed roughly the same during the 4-day exposure (Figure 4). Only AHTN at the highest test dose showed a significant increase at day 3. In fish exposed to 0.1 μM AHTN, about 15.8 mg/kg fw (i.e., ∼61 μM) was found, while fish exposed to 1 μM AHTN contained about 143 mg/kg fw (i.e., ∼554 μM). Fish exposed to 0.1 μM HHCB contained approximately 18.9 mg/kg fw (i.e., ∼73 μM) and fish exposed to 1 μM HHCB about 135 mg/kg fw (i.e., ∼523 μM). This means that the internal concentration is about a factor 600 higher than the nominal test dose.

Discussion

In this study we were mainly interested in the in vitro and in vivo antiestrogenic effects of two polycyclic musks (AHTN and HHCB), which are ubiquitously present in the aquatic environment. For the in vitro experiments, we used the ERα and ERβ subtypes cloned from human and zebrafish, and also the novel zebrafish ERγ (*9*). Next to ERα and ERβ, fish have a third ER subtype (ERβ2 or ERγ), which has been recently discovered in the Atlantic croaker (*17*), goldfish (*18*), and the zebrafish (*10, 11, 19*; see also Callard et al., unpublished results; GenBank Nos. AAK16740, AAK16741, and AAK16742). Comparison of the overall amino acid sequences of the three receptors indicates that each zfER protein was generated by a distinct gene (*11*). Alignment of

the ligand binding domains of the human and the zebrafish ERα reveals a homology of 55%. The percentage of identical amino acid residues of the human and the zebrafish ERβ is 57%, and that of hERβ and zfERγ is 61% (*11*). These alignment results taken together with phylogenetic analysis clearly indicated that zfERα belongs to the ERα subgroup and that both zfERβ and zfERγ belong to the ERβ subgroup (*11*).

The zebrafish ER subtypes, which have been cloned in our laboratory, have been shown to bind estradiol in a receptor-binding assay (Zeinstra et al., manuscript in preparation). This was partly to be expected, since all three zebrafish ERs contain the same specific amino acids for estradiol binding as are found in the human ERα, i.e., Glu353, Arg394, and His524 in hERα (*20*). In the present results we show that zebrafish ERγ is the most sensitive to estradiol, which was also shown in other studies (*9*, *11*). Furthermore, the difference between E2-stimulated transactivation of human ERα and ERβ was not shown in the zebrafish situation, where ERα and ERβ are about equally potent and ERγ is the most responsive transactivator. Overall, zebrafish ERα and ERβ are less sensitive to E2 than their human counterparts, which also has been shown for rainbow trout ERs (*21*). This may explain the fact that both musk compounds do not show any agonistic effect on the three zebrafish ERs. On the human ERα, the transactivation potential of which is minimally a factor of 10 higher than those of the zebrafish ERs, a marginal agonism has been shown in stably transfected HEK293 cells (*5*). On the other hand, antiestrogenic effects of AHTN and HHCB on the human ERs have been recently shown (*5*). In the present study we also showed a dose-dependent antagonism on zfERγ, already starting at 100 nM and which is almost as strong as that on hERβ. Of the three zebrafish ERs, zfERγ showed the highest susceptibility for suppression of E2 induction by OHT and both musks.

In our in vivo transgenic zebrafish study, at test concentrations of 0.1 and 1 μM AHTN or HHCB, a significant repression of E2-induced transactivation was observed, especially at the EC50 E2 concentration. This repression is quite strong, namely, down to 20% of the E2 induction for both compounds at the highest test dose.

The nominal water concentrations used in this in vivo study (0.01, 0.1, and 1 μM musk, i.e., 2.58, 25.8, or 258 μg/L musk) were comparable to the nominal concentrations used in standard fish toxicity studies. In a 36-day early-life-stage test (OECD 210) with fathead minnow (*Pimephales promelas*) no observed effect concentrations (NOECs) were found of 35 μg of AHTN/L and 68 μg of HHCB/L. In a 21-day growth test (OECD 204) with bluegill sunfish (*Lepomis macrochirus*) NOECs were found of 89 μg of AHTN/L and 93 μg of HHCB/L (*22*). The actual concentrations at which we observe antiestrogenic effects are around or below the no-observed-effect levels from these studies. That is, no developmental disorders were or will be observed at the concentrations used in our transgenic zebrafish assay.

Furthermore, the nominal concentrations at which an antiestrogenic effect is observed are roughly 25 to more than 1000 times higher than the concentrations found in the environment. Under normal environmental conditions, in river and seawater, levels of musk in the nanogram per liter range are found (reviewed in refs *23* and *24*). The higher the input of sewage water, the higher the concentration of musk that is found (*3*). Maximum levels in the low microgram per liter range have been found in water samples from lakes with a high input of sewage water. Higher concentrations have been found in sewage water effluents and sewage settlement ponds, with maximal levels in eel of 19.2 mg/kg fw (cited in ref *25*). In eel under natural conditions mean levels of 43 μg/kg fw were found (cited in ref *3*). In other fish species under natural conditions, comparable or lower concentrations are found, depending largely on the lipid content (reviewed in refs *23* and *24*). The internal concentration that we have found at the lowest observed effect concentration for antiestrogenicity in vivo (i.e., 0.1 μM) is about 15.8 mg of AHTN/kg fw and 18.9 mg of HHCB/kg fw. These concentrations can be reached in fish from ponds of sewage treatment plants (*24*, *25*) and might induce antiestrogenic effects in this particular situation. Under normal environmental conditions, the concentrations of the musks in receiving waters are too low to cause antiestrogenic effects.

Concentrations in the fish depend on the toxicokinetics and the biotransformation of the compound in the fish. The high antagonistic potency in vivo may be due to its high lipophilicity and thus to its strong bioaccumulation. From our GC−MS results it is clear that both musks are indeed bioaccumulating. In the zebrafish, concentrations have been found which are roughly a factor 600 higher than the nominal test dose. Internal concentrations stay roughly the same during the 96 h in vivo experiment, which means that the equilibrium has been reached within 1 day. Butte et al. (*26*) showed that for both polycyclic musks the steady state for uptake in zebrafish was reached in about 10 h. Concentrations in the fish also depend on the fate of the compound in the aquarium. When AHTN or HHCB is added to beaker glasses without fish, immediately after the first dosing, 15−30% has disappeared, and at the end of the 96 h experiment, 25−40% has disappeared. Compounds may sorb to the glass wall and detritus, or degradation of the compounds may take place. HHCB can be transformed into HHCB lactone by autoxidation, which can also occur as a biotransformation reaction (*27*). Nevertheless, a significant amount of musk has been accumulated in the fish, resulting in low actual water concentrations at the end of the exposure experiment, and also in the observed antiestrogenic effects.

The antiestrogenic effects seen in the in vivo transgenic zebrafish assay are probably for the greater part mediated by the zfERγ, which is the most responsive to estradiol in our in vitro assays. Also, the strongest antagonism by the musks was seen at this receptor subtype. However, it cannot be ruled out that the other two ER subtypes also play a role in the netto effect. Comparison between the in vitro system and the in vivo test system is possible because the measured end point (luciferase protein) is induced according to the same principle. In both assays the compound binds to the endogenous ER and activates it. Then the ER−ligand complex binds to EREs present on the luciferase target gene, which is introduced in the genome of both the cells and the fish. Finally the luciferase protein is induced. To our knowledge, this is the first time that environmental contaminants are shown to be antiestrogenic in an in vivo fish assay. In scientific literature, antiestrogenic effects have been observed in cultured fish hepatocytes by measuring the concentration or expression of the estrogen-regulated yolk protein vitellogenin (Vtg) (*28*, *29*). Letcher et al. (*29*) showed the antiestrogenic effects of PCB metabolites in an in vitro carp hepatocyte assay by measuring Vtg, and made clear that these effects were ER-mediated, rather than aryl hydrocarbon receptor (AhR)-mediated. Monteverdi and Di Giulio (*30*) and Latonnelle et al. (*28*) observed antiestrogenic effects of tamoxifen in, respectively, channel cat fish and Siberian sturgeon hepatocyte cultures, by showing repressed Vtg synthesis. These effects were also postulated to be ER-regulated. However, these studies are in vitro studies, lacking important aspects of in vivo functioning, such as kinetics and biotransformation.

Several in vivo studies looked at the downregulation of Vtg synthesis by antiestrogenic AhR agonists. AhR agonism is assessed by measuring CYP1A induction. TCDD and nonortho PCBs function via an AhR-mediated mechanism of action, involving cross-talk between the ER and the AhR (*31*). In an in vivo study by Arukwe and collegueas (*32*), the

antiestrogenic CYP1A inducer 3,3′,4,4′-tetrachlorobiphenyl (TCB) shows both potentiation and reduction of nonylphenol-induced Vtg synthesis, depending on doses and temporal exposure sequence. Another in vivo study shows both potentation and repression of E2-induced Vtg synthesis by the CYP1A inducer β-naphthoflavone, which has been shown to be antiestrogenic in vitro (*33*). These studies assessed antiestrogenicity which is both ER- and AhR-mediated. Only a few in vivo fish studies showed antagonism that was postulated to be solely ER-dependent. Tilapia showed decreased E2-induced Vtg levels after tamoxifen exposure for 12 days (*34*). Similar results were found in exposed female Tilapia by Lazier et al. (*35*). Recently, Panter et al. (*36*) exposed fathead minnows via the water. However, no dose-dependent antagonism was found when fish were exposed to ethinylestradiol and the antiestrogen ZM189,154. Using an AhR-specific CALUX assay, we showed no in vitro (ant)agonistic effects of the musks on the human AhR (unpublished data). Furthermore, AHTN is not a CYP1A inducer (*37*). We conclude that, in our in vivo assay, the observed antiestrogenic effects are not AhR-mediated, but solely ER-mediated. This makes the transgenic zebrafish assay a suitable tool for the rapid detection of both estrogenic and antiestrogenic effects of chemicals in an in vivo fish model.

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